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STUDIES ON SHIGELLA EQUIRULIS

(BACTERIUM VISCOSUM EQUI)

BULLETIN NO. 320.

(RESEARCH BULLETIN)

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BULLETIN NO. 320.

(RESEARCH BULLETIN)

Studies on Shigella Equirulis (Bact. viscosum equi.)

By PHILIP R. EDWARDS

Part 1. A Comparative Study of 40 Strains.

For 15 years *Shigella equirulis* has been recognized in Europe as a serious factor in septicemia, arthritis and nephritis of young foals. It is now known to be responsible for the early death of a larger number of foals than any other individual microorganism. Foals infected with this bacterium frequently die within 18 hours. The average age at death as reported by Dimock, Edwards and Bullard [1] is 2.9 days. The organism is found in infected verminous aneurysms of older foals and occasionally produces death in adult horses. For a detailed description of the symptoms and pathology resulting from this infection the reader is referred to the paper cited.

The presence of *S. equirulis* in America has only recently been recognized. Good and Smith[2] working on joint ill in foals, isolated an organism which from their description was undoubtedly *S. equirulis*. Snyder[3] described four cases of infection due to this organism and for the first time called attention to the presence of the bacterium in this country. Dimock, Edwards and Bullard[1] isolated *S. equirulis* from 33 foals. The observations cited were made at the Kentucky Agricultural Experi-

[1] Dimock, W. W., Edwards, P. R., and Bullard, J. F. 1928. Bact. viscosum equi: a factor in joint ill and septicemia of young foals. Jour. Amer. Vet. Med. Assoc. *73*, 163.

[2] Good, E. S., and Smith, W. V. 1914. Bacillus abortivo-equinus as an etiological factor in infectious arthritis of colts. Jour. Infect. Diseases, *15*, 347.

[3] Snyder, E. M. 1925. Eberthella viscosa (Bact. viscosum equi): Etiological factor in joint ill. Jour. Amer. Vet. Med. Assoc. *66*, 481.

ment Station. Since these publications appeared Udall, Fincher, and Gibbons[4] have recorded the isolation of the organism in New York, and Bullard[5] has recovered it from foals in Indiana.

Only slight attention has been given *S. equirulis* from a bacteriological standpoint altho much has been written concerning the method of infection, symptoms, pathological changes, and treatment of the disease produced by the microorganism. Several publications have appeared in which the cultural and biochemical characters of the bacterium have been studied but no results of a systematic study of a number of strains have been published. Study of the serological characters of the organism has been even more neglected. For this reason it was thought worth while to collect a number of strains and study their cultural, biochemical and serological properties.

Many names have been used for this species. The first name given the organism was *Bacillus nephritidis equi,* by Meyer. The second was *Bacillus equirulis,* by van Straaten. Magnusson applied a third name to the bacterium, calling it *Bact. viscosum equi.* Bergey[6] has recently classified the organism as *Shigella viscosa.* Since *Bacillus nephritidis equi* is a trinomial which in a strictly grammatical sense cannot be hyphenated, it seems that the valid name for this species is *Shigella equirulis* (van Straaten). The synonyms which have been encountered are as follows:

Bacillus nephritidis equi Meyer, Transvaal Dept. Agr., Rpt. of the Govt. Bacteriologist, 1908-9- 122-163.

Bacillus equirulis van Straaten, Verslag van de Werksaamheden der Rijksseruminrichting, 1916-17.

Bact. viscosum equi Magnusson, Svensk. Veterinärtijdskr, 1917, 81.

Bacillus pyosepticus equi de Blieck u. van Heelsbergen, Tijdschr. v. Diergeneesk,, 1919, *46*, 492.

Bact. pyosepticum viscosum Miessner, Deut. tierärtzl. Wchnschr., 1921, *29*, 185.

Bact. pyosepticum visosum equi Lütje, Deut. tierärtzl. Wchnschr., 1921, *29*, 185.

Bact. pyosepticum (viscosum) Miessner u. Berge, Deut. tierärtzl. Wchnschr., 1922, *30*, 473.

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[4] Udall, D. H., Fincher, M. G., and Gibbons, W. J. 1929. Bact. viscosum equi infection in a foal. Cornell Vet., *19*, 25.

[5] Bullard, J. F. 1930. Bact. viscosum equi infections in foals of the heavy breeds. Jour. Amer. Vet. Med. Assoc., *76*, 250.

[6] Bergey, D. H. 1930. Manual of Determinative Bacteriology, third edition.

Bact. pyosepticum Miessner, Deut. tierärtzl. Wchnschr., 1923, *31*, 348.

Bact. pyosepticum equi Laudien, Inaug. Diss., Hannover, 1923.

B. pyosepticus Clarenberg, Ztschr, f. Infekkr. u. Hyg. d. Haust., 1924, *27*, 193.

Bact. equi Weldin and Levine, Abstr. Bact., 1923, *7*, 13.

Eberthella viscosa Snyder, Jour. Am. Vet. Med. Assoc., 1925, *66*, 481.

Shigella equi Weldin, Iowa State Col. J. Sci., 1927, *1*, 121.

Shigella viscosa (Magnusson) Snyder, Bergey, et al., Manual of Determinative Bacteriology, 3rd edition, 1930, 363.

The cultures used in this work consisted of a group of 40 strains of *S. equirulis* isolated from young horses which on post-mortem examination exhibited lesions characteristic of the infection. The designations of the cultures and the sources from which they were isolated are given in table 1.

Table 1. Sources of Cultures.

Designation	Source
26	Recovered from fetal membranes, foal dead at birth.
2, 3, 4, 5, 6, 8, 9, 10, 11, 13, 23, 24, 25, 27, 28, 29, 31, 32, 34, 35, 36, 37, 38, 39	Isolated from organs of foals dead at 1–4 days of age.
1, 12, 30	Isolated from organs of foals dead at 5–10 days of age.
7, 33	Isolated from organs of foals dead at 11–42 days of age.
14, 15, 16, 17, 18, 19, 20, 21, 22, 40	Isolated from organs of foals dead at age of more than 42 days.*

* The ages of these foals varied from 60 to 240 days. The majority had infected verminous aneurisms of the mesenteric artery. Two had chronic lesions in the umbilical vein, indicating that they were infected at an early age.

Morphology and Staining. The organisms in all the cultures were non-motile rods conforming to the generic description (*Shigella, Bergey,* et al, 1930). The organisms are highly pleomorphic; single rods, streptococcus-like chains, and long filamentous forms occur in the same smear. When young

(16-20 hours) the organisms stain readily with the anilin dyes but as the age of the cultures increase the majority of the bacteria stain with difficulty. Tho several workers have described this species as a capsule former; we have not been able to demonstrate capsule formation in any of our cultures.

Cultural Characteristics. The organism grows readily on the ordinary culture mediums. Growth is more profuse on mediums containing fresh beef infusion than on those prepared from meat extract. The bacterium may be cultivated in either of two forms, the rough or the smooth, these adjectives being descriptive of the colonies produced.

Since the rough form usually is isolated from the organs of infected individuals, it will be described here. The cultural characters of this form are as follows:

The most prominent character of the organism when growing in any medium in its production of large amounts of slime; it is extremely mucoid. This property, however, should not be confused with the mucoid characters of the Friedländer group. Colonies of Friedländer bacilli are usually very moist, mucoid, and spreading, and string out into long threads when touched with a needle. *S. equirulis* produces colonies which appear to have a dry, dull surface. These colonies when touched with a needle do not string out into long threads but resist transfer. A loop may be drawn across the surface of the colony and considerable pressure exerted without a perceptible amount of growth being picked up or the surface of the colony visibly disturbed. Well isolated agar colonies attain considerable size (3–5 m. m. diameter) in 48 hours. These are raised and opaque. At times the colonies may appear almost hemispherical. The edges of the colonies are undulate and the surface extremely rough. When examined under a hand lens by reflected light they present a lobulated appearance, the small lobular projections appearing over the entire surface of the colony. Between the lobules deep crevices appear. When viewed by transmitted light the roughened surface causes the colony to exhibit an irregular radially striated structure. With increasing age a clear, smooth border is extended from the colony. This gives it

the appearance described by Magnusson[7] who says of the colonies: "They are of a mucoid, glossy, gray color and under the microscope have a darker nucleus and a wide clear border-zone".

On agar slants a rather opaque, grayish-white growth appears. This is extremely tenacious and is removed from the agar with difficulty. The growth is not spreading and there is little tendency to colonization. One of the most marked peculiarities of the organism is exhibited when it is grown on the surface of agar. On this medium the organism does not remain viable for more than eight or ten days when kept in the dark at room temperature. If exposed to sunlight its life is even shorter. In keeping stock cultures the practice has been to grow them on fresh beef infusion agar and transfer them every sixth day. In this way 15 cultures were maintained for more than three years. Even when this precaution is taken, a culture is occasionally lost. In broth the organism remains viable for two to four weeks. In gelatin and chopped meat medium the bacteria live for even longer periods.

In broth a very characteristic growth appears. The first growth to become apparent is a collection of small masses of bacteria on the side of the culture tube. A few masses also may be observed floating on the surface of the medium. As growth continues a light pellicle of uneven thickness is formed on the surface of the broth and the sides of the tube are covered with a deposit of the bacteria. A ropy sediment containing granules appears and eventually a diffuse growth occurs thruout the liquid. Broth cultures become very viscous and may string out in threads several inches in length when a needle is placed in the culture and slowly withdrawn.

In gelatin at 20° C a nail-head growth occurs. In gelatin incubated at 37° C small colonies develop thruout the tube. Liquefaction has not been observed in any of the cultures.

Biochemical Characters. The cultures have been tested for nitrate reduction, ammonia production, growth in uric acid and

[7] Magnusson, H. 1919. Joint Ill in Foals: Etiology. Jour. Compar. Path. and Therap., *32*, 143.

citrate mediums, indol production, reaction to the methyl red and Voges-Proskauer tests, and behavior in litmus milk. Only one of the forty cultures, strain 20, gave a positive methyl red test. The growth of the organism in the dextrose-phosphate broth used in the tests was very poor. None of the organisms produced acetyl-methyl-carbinol and none were able to initiate growth in uric acid or citrate mediums. Indol was not produced by any of the cultures. All strains reduced nitrate to nitrite and all produced slight but definite amounts of ammonia. Permanent acidity was produced in litmus milk by all strains. In addition 10 of the cultures, strains 7, 10, 12, 13, 14, 20, 21, 23, 34 and 35, caused coagulation with reduction of the litmus.

Fermentative Reactions. We have tested the ability of the 40 strains to form acid and gas from glucose, levulose, galactose, maltose, sucrose, lactose, arabinose, xylose, rhamnose, raffinose, glycerol, dulcitol, sorbitol, mannitol, adonitol, salicin, inulin, dextrin and starch. Solutions of the fermentable substances were sterilized by filtration and added to previously sterilized broth in amounts sufficient to give a final concentration of one percent. Durham fermentation tubes were used to observe gas production and Andrade's indicator to detect acid production. Incubation was continued 14 days after inoculation if acid production was not observed earlier. The results of the fermentation tests are given in table 2.

No gas production was observed in any of the tests. From table 2 it may be seen that all cultures produced acid from glucose, levulose, galactose, maltose, lactose, sucrose, xylose, raffinose and mannitol. No acid was produced by any of the cultures in broth containing rhamnose, dulcitol, and sorbitol. Five of the cultures, 3, 19, 20, 24, and 29, produced acid from arabinose while the other cultures did not attack it. Glycerol was fermented by the majority of the strains but six, 7, 9, 14, 17, 20, and 27, produced no acid in glycerol broth. Adonitol was fermented by eight of the cultures, 3, 4, 6, 14, 15, 19, 28 and 29, while the remainder formed no acid from this substance. Salicin was attacked by only four of the strains, 9, 12, 20 and 26. Only two of the cultures, 12 and 20, produced acid from inulin. Acid

was formed from dextrin by all but 3 of the cultures, 1, 6, and 11 failed to ferment this substance. Starch was fermented by only five cultures, 3, 4, 12, 20, and 29. In most instances in which fermentation was observed, acid production was prompt and distinct. Only in the fermentation of glycerol and adonitol was late acid production recorded.

Serological Reactions. In investigating the serological characters of the organisms, agglutination, precipitin and agglutinin absorption tests were employed. Rabbits were used in the preparation of agglutinating serums. Living organisms were injected intravenously on three successive days followed by a rest period of 4 days. The amount of bacilli was increased gradually until 2.5cc of a heavy suspension was given at each injection. In general the organisms are rather low in agglutinogenic power. Fifteen to eighteen injections usually were required before a satisfactory titer was reached. Seven strains, 2, 3, 5, 16, 20, 36 and 38, were used to prepare serums. Using these seven serums agglutination tests were set up, all of the 40 clutures were used as antigens. The dilutions employed in the tests ranged from 1 to 20 to 1 to 2000. All tests were incubated 24 hours at 37° before reading. They were then allowed to stand at room temperature an additional 24 hours and read again. The results of the tests are given in table 3.

The results of the tests are extremely irregular. Each serum agglutinated its homologous strain in the highest dilution employed. Several cultures, 1, 9, 11, 15, 24, and 29 were more or less agglutinable in normal serum. These cultures were agglutinated in fairly high dilution by all the antiserums. Strain 23 was agglutinated in high dilution by serums 2 and 36 but not by the other serums. Strains 6, 32, and 34 were agglutinated in high dilution by serums 38, 5 and 36 respectively but were acted upon only in low dilution or not at all by the remaining serums. The remainder of the cultures were agglutinated in low dilution or not at all.

Precipitin Tests. When *S. equirulis* is grown in liquid cultures it elaborates a soluble specific substance which is highly reactive with antiserum. This substance serves admirably as

antigen in the precipitin test. It has been found that the specific substance can be obtained in greater concentration and with less contamination of other reactive substances if the organisms are grown on agar slants and washed off with physiological saline. The saline suspensions are autoclaved at 15 pounds pressure for 15 minutes and passed thru Berkefeld filters to free them of bacteria. The resulting clear filtrates are used as antigens in the precipitin tests. These antigens are capable of causing precipitation in amounts as small as .01cc.

All the 40 cultures were used to prepare antigens for the precipitin tests. Each of these antigens was titrated against the seven serums used in the agglutination tests. The amount of serum used in each test was 0.1cc. Antigens were used in amounts varying from 1.0cc. to 0.01cc. The tests were incubated at 55° for one hour, read, placed in the ice chest over night and a second reading taken the following morning.

The results of the precipitin tests were clear-cut and confirmed the results obtained by agglutination. Each serum gave marked precipitation with its homologous antigen. In addition to the reactions obtained with homologous serums and antigens, antigen 6 gave marked precipitation with serum 38, antigen 32 reacted strongly with serum 5, and antigen 34 gave heavy precipitation with serum 36. With the exception of these reactions all the tests were negative. The cross reactions which occurred in the lower dilution of the agglutination tests were not apparent here.

Agglutination Absorption. Each of the seven agglutinating serums was absorbed with the 40 cultures being studied. The absorptions were carried out using the method of Edwards and Rettger[8]. The results of these tests confirm the results obtained in the agglutination and precipitin tests. Each of the strains used to prepare serum completely exhausted that serum of agglutinins. Strain 6 removed agglutinins completely from serum 38; strain 32 exhausted serum 5 of agglutinins for its homologous strain, and strain 34 caused a complete removal of agglu-

[8] Edwards, P. R., and Rettger, L. F. 1927. The paratyphoid B-suipestifer group of bacteria. Jour. Bact., *13*, 73.

tinins from serum 36. The remaining 30 strains did not absorb agglutinins from any of the serums nor did they materially lower the titer of the serums for their homologous organisms.

DISCUSSION

The study of 40 cultures isolated from foals that died with symptoms and lesions typical of *S. equirulis* infection has shown that the organisms are similar in morphology, cultural characters, and biochemical reactions. All the strains exhibit a highly variable morphology, streptococcus-like chains, short rods and long filamentous forms being found in the same smear. The cultural characters have been found to vary, due to disassociation of the bacilli. A more complete description of the morphological and cultural variations is given in Parts 2 and 3 of this Bulletin.

Altho some minor differences were observed in the fermentative characters of the bacilli, the biochemical characters are comparatively constant. The results obtained in the fermentation tests agree in large part with those obtained by Magnusson[7] who reported that the organism produced acid from glucose, lactose, sucrose, maltose, raffinose, mannitol, and galactose. Arabinose, rhamnose, adonitol, and dulcitol, he found, were not attacked. This worker mentioned no irregularities in the results of his tests. However, he did not state how many cultures were used in testing the fermentative characters of the organism so that it is impossible to base any opinion as to the constancy of the fermentative activities of this species on his work. Separation of the bacteria on the basis of their action on sugars does not seem justified.

The action of the cultures on milk is of interest as different investigators have disagreed as to the action of the microorganism on this substance. Magnusson[7] stated that milk was not coagulated. Reinhardt[9] also reported that the bacterium did not

[9] Reinhardt. 1921. Beitrag zur Aetiologie der Fohlenlähme. Monatsch. Prakt. Tierheilk., *32*, 154.

coagulate milk. Lütje[10 11 12] emphasized the fact that milk was not coagulated. He states that the viscosity of the milk cultures may be deceiving. deBlieck and Baudet[13], Iwanoff[14], Miessner and Berge[15] stated that *S. equirulis* coagulated milk. Clarenburg[16] stated that freshly sterilized milk was coagulated while milk that had stood for some time was coagulated very slowly or not at all. Goerttler[17] confirmed Clarenburg's observations. Miessner and Wetzel[18] in their review of the subject accepted the views of Clarenburg and Goerttler.

We have repeatedly tested the action of the 40 cultures on milk which was sterilized and inoculated immediately after being drawn and on milk which had stood several days after sterilization. The tests were performed both in the presence and absence of litmus. The results have been the same in every case. Standing after sterilization apparently had no effect on the coagulability of the milk. The majority of our cultures always failed to bring about a coagulation of milk, but twenty-five percent of the strains coagulated the milk in every test.

Little work has been done on the serological characters of *S. equirulis.* Lütje[11] noted that marked differences existed in the agglutination of different strains with a polyvalent serum prepared for therapeutic purposes. On the basis of these results he suspected that the strains were not serologically identical.

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[10] Lütje, F. 1921. Fohlenkrankheiten. Deut. tierärtzl. Wchnschr., *29*, 463.

[11] Lütje, F. 1922. Infektionen mit dem Bact. pyosepticum (viscosum) equi. Deut. tierärtzl. Wchnschr., *30*, 4.

[12] Lütje, F. 1925. Weitere Fälle einer Infektion mit dem Bacterium pyosepticum (viscosum) equi bei Ferkeln. Deut. tierärtzl. Wchnschr., *33*, 129.

[13] de Blieck und Baudet. 1920. Ein wichtiger Bacillus als Ursache der Septicopyämie beim Fohlen. Deut. tierärtzl. Wchnschr., *28*, 57.

[14] Iwanoff. 1921. Uber das kulturelle und pathogene Verhalten des Bact. pyosepticum viscosum. Inaug. Diss. Hannover.

[15] Miessner, H. und Berge, R. 1922. Verfohlen und Fohlenkrankheiten. Deut. tierärtzl. Wchnschr., *30*, 473.

[16] Clarenburg, A. 1924. Bacillus pyosepticus als Krankheitsurache bei einem Schwein. Ztschr. Infektionskrank. u. Hyg. Haustiere, *27*, 193.

[17] Goerttler, V. 1926. Kritische Prüfung des bakeriologischen Merkmales des Bact. pyosepticum viscosum equi. Centbl. Bakt. (Etc) I, Orig., *98*, 227.

[18] Miessner, H., u. Wetzel, R. 1929. Infektiose Aufzuchtkrankheiten der Tiere. Kolle u. Wassermann, Handb. d. path. Mikroorg. 3 Aufl. *6*, 605.

Laudien[19] prepared an agglutinating serum and found that while it agglutinated some strains of the organism in a dilution of 1:2000, other cultures were not agglutinated in dilutions higher than 1:200. Kowatsch[20] found marked differences in the serological properties of a number of strains. However, the cultures with which Kowatsch worked exhibited such marked differences in cultural and biochemical characters that Goerttler[21] justifiably has questioned the purity and identity of his cultures Goerttler[21] investigated the agglutinative properties of a number of cultures of *S. equirulis*. He found that saline suspensions of agar cultures were unsuitable for use as antigens because of the viscosity and inagglutinability of the organisms. In order to overcome this difficulty Goerttler treated the bacteria with 66% alcohol for 2 to 3 hours, centrifuged, and resuspended the organisms in carbolized saline. This treatment rendered the organisms more agglutinable, but was not sufficient to make all the strains agglutinable. Goerttler noted a close correlation between the agglutinability and agglutinogenic powers of his various cultures. Those strains which were easily agglutinable readily produced agglutinins upon injection into rabbits and horses, whereas inagglutinable strains caused little or no rise in the agglutinative titers of the serums of the treated animals. Seven strains which were agglutinable were found to be agglutinated in more or less high dilution by a single serum. While Goerttler emphasized the quantitative differences in agglutinability and agglutinogenic properties noted above, he found no qualitative antigenic differences and apparently is of the opinion that the organisms are all closely related serologically. Many of his cultures were agglutinated in quite high dilution in normal serum. His results in general are unconvincing.

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[19] Laudien. 1923. Kotuntersuchungen bei Pferden auf die Anwesenheit von Bact. pyosepticum viscosum equi und von Paratyphusbacillen. Inaug. Diss. Hannover.

[20] Kowatsch. 1925. Zur Morphologie und Biologie des Bact. pyosepticum equi. Deut. tierärtzl. Wchnschr., *33*, 143.

[21] Goerttler, V. 1925. Die Agglutinationsverhältnisse des Bact. pyosepticum equi und der Agglutiningehalt normaler Pferdeseren gegenüber diesem Bakterium. Deut. tierärtzl. Wchnschr., *33*, 545.

In the present work it has been found that the organisms form an extremely heterogeneous serological group. The results obtained by agglutination, precipitation and agglutinin absorption are in close agreement. Using seven agglutinating serums we have noted only 3 cultures that were identical with any of the seven strains. Strain 6 is serologically identical with strain 38, strain 5 with strain 32, and strain 34 with strain 36. In addition to these 3 cases of identity there is a certain amount of cross agglutination in low dilution in the tests. The author is of the opinion that these are group reactions. They did not appear in the precipitin tests and cultures agglutinating in low dilutions with one or more of the serums were unable to materially lower the titers of the serums when used as antigens in absorption tests. We have repeated Goerttler's work using alcohol-treated antigens. So far as we have been able to observe, alcohol treatment has no effect on the agglutination test. In addition to the seven serums described in this paper serums were prepared for 8 other strains. In these 15 cultures used to prepare agglutinating serums no culture was encountered which was inagglutinable or which failed to produce agglutinins in the treated animal. We have not been able to confirm the observations of Goerttler on agglutinability and agglutinogenic powers.

Many workers, notably Langhoff,[22] have attempted to diagnose infection with *S. equirulis* in cases of abortion and to determine whether the infection in foals was of prenatal origin by using the serum of the dam in agglutination tests. Langhoff has claimed that the infection may be diagnosed in this way. According to the results of our studies it would be impossible to determine the presence of agglutinins in the serum unless the infecting organisms were isolated and used as antigen in the tests.

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[22] Langhoff, L. 1923. Infektion mit dem Bakterium pyosepticum viscosum equi unter besondere Berüksichtigung der makroskopischen pathologisch-anatomischen Erscheinungen. Berlin. tierärtzl. Wchnschr., *39*, 358.

Table 2. **Fermentation Tests**

Culture	Glucose	Levulose	Galactose	Maltose	Sucrose	Lactose	Arabinose	Xylose	Rhamnose	Raffinose	Glycerol	Dulcitol	Sorbitol	Mannitol	Adonitol	Salicin	Inulin	Dextrin	Starch
1	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	O	O
2	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
3	A	A	A	A	A	A	A	A	O	A	A	O	O	A	A	O	O	A	A
4	A	A	A	A	A	A	O	A	O	A	A	O	O	A	A	O	O	A	A
5	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
6	A	A	A	A	A	A	O	A	O	A	A	O	O	A	A	O	O	O	O
7	A	A	A	A	A	A	O	A	O	A	O	O	O	A	O	O	O	A	O
8	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
9	A	A	A	A	A	A	O	A	O	A	O	O	O	A	O	A	O	A	O
10	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
11	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	O	O
12	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	A	A	A	A
13	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
14	A	A	A	A	A	A	O	A	O	A	O	O	O	A	A	O	O	A	O
15	A	A	A	A	A	A	O	A	O	A	A	O	O	A	A	O	O	A	O
16	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
17	A	A	A	A	A	A	O	A	O	A	O	O	O	A	O	O	O	A	O
18	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
19	A	A	A	A	A	A	A	A	O	A	A	O	O	A	A	O	O	A	O
20	A	A	A	A	A	A	A	A	O	A	O	O	O	A	O	A	A	A	A
21	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
22	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
23	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
24	A	A	A	A	A	A	A	A	O	A	A	O	O	A	O	O	O	A	O
25	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
26	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	A	O	A	O
27	A	A	A	A	A	A	O	A	O	A	O	O	O	A	O	O	O	A	O
28	A	A	A	A	A	A	O	A	O	A	A	O	O	A	A	O	O	A	O
29	A	A	A	A	A	A	A	A	O	A	A	O	O	A	A	O	O	A	A
30	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
31	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
32	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
33	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
34	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
35	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
36	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
37	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
38	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
39	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O
40	A	A	A	A	A	A	O	A	O	A	A	O	O	A	O	O	O	A	O

A - acid production
O - no acid production

Table 3. Agglutination

	SERA							
Antigens	2	3	5	16	20	36	38	Normal
L	1000	200	500	500	200	500	500	200
2	2000	0	50	50	50	0	0	0
3	20	2000	20	50	20	0	0	0
4	20	0	0	50	50	20	20	0
5	0	0	2000	0	50	20	0	0
6	20	100	0	20	0	50	1000	0
7	100	50	0	50	50	20	0	0
8	50	0	20	20	50	50	50	0
9	50	20	20	20	0	20	50	20
10	50	0	20	20	50	50	20	0
11	50	50	50	50	50	50	50	50
12	0	20	20	0	0	20	0	0
13	200	0	50	0	50	20	0	0
14	20	50	20	20	0	50	0	0
15	200	50	20	200	200	200	100	100
16	20	0	0	2000	0	0	0	0
17	0	0	0	0	0	0	0	0
18	100	0	0	50	100	100	50	0
19	0	0	0	50	20	50	0	0
20	0	0	0	0	2000	0	0	0
21	0	0	0	20	20	20	0	0
22	100	0	0	50	50	20	50	0
23	1000	0	0	0	100	1000	0	0
24	1000	200	1000	200	200	1000	200	200
25	0	0	0	0	0	50	0	0
26	0	0	0	0	0	20	0	0
27	50	0	0	50	50	50	20	0
28	100	0	0	50	0	20	0	0
29	1000	100	1000	500	1000	1000	1000	1000
30	0	0	0	0	0	0	0	0
31	100	0	20	20	50	100	50	0
32	50	0	2000	50	0	0	0	0
33	50	20	50	0	100	0	0	0
34	50	0	50	20	50	2000	0	0
35	0	0	0	0	0	200	0	0
36	0	0	0	0	0	2000	20	0
37	0	50	0	0	0	20	50	0
38	50	0	0	0	0	50	2000	0
39	0	100	0	0	0	50	50	0
40	0	0	0	0	0	0	0	0

0 - indicates no agglutination at 1-20
Figures indicate highest dilution in which agglutination occurred.

Part 2. Rough and Smooth Variants.

While studying the characters of a number of strains of *Shigella equirulis* the writer was impressed with the marked variation in cultural characters taking place in these organisms. Other workers have noted variation in this species but no general agreement has been reached as to the extent and character of the changes. These writers have been interested primarily in mucoid and non-mucoid forms of the bacterium and have limited their statements concerning variation in this species to this property. In doing so they failed to mention the most striking change that takes place in the cultures, namely, the transition from rough to smooth colonies. Changes in the mucoid properties of the bacterium are very closely correlated with the change of the colonies from the rough to the smooth form and it is possible to arrive at a better understanding of the changes if the two characters are considered together. For brevity, the adjectives, rough and smooth, are used in this bulletin to distinguish the organisms of the two strains, and their attributes.

Lütje[10] [11] noted that the bacteria, when originally isolated, formed tough, adherent colonies but that after continued culture the organism produced a more moist, mucoid type of colony. These, on further culture, lost their tenacity entirely. Clarenburg[16] observed that when the bacilli were cultivated artificially the mucoid character of the growth gradually was lost. Beller[23] also noted the loss of mucoid properties on continued culture. Goerttler[17] on the contrary found little if any deviation in the viscosity of 20 strains of the organism. He found the mucoid character so constantly that he states that when this property is lacking in a culture its identity must be questioned.

If the presence of rough and smooth variation and its relation to mucoid and non-mucoid forms is recognized it may be seen that variation in this species is more or less orderly and the

[23] Beller. 1924. Zur geographischen Verbreitung, Aetiologie, und Bekämpfung der Fohlenpyoseptikämie. Müenchen. tierärtzl. Wchnschr., *75*, 181.

same changes take place in the majority of the strains of the organism. *S. equirulis,* in most instances, is isolated as a rough, mucoid type. As cultivation is continued the roughness of the colonies gradually disappears and eventually the bacterium produces smooth colonies. With this change in colony form the mucoid property is gradually lost. When first isolated, the colonies are tough, rather dry, and very adherent. This dry, tenacious form gradually becomes more moist and stringy so that it will draw out in long threads when touched with a needle. This stage constitutes the smooth, mucoid form. As artificial culture is continued the mucoid propertes are lost until finally the culture becomes a smooth non-mucoid race. Roughness and tenacity are closely associated. In the isolation and cultivation of more than 100 strains of *S. equirulis* a rough culture has never been observed which did not produce tough, adherent colonies. Conversely, non-mucoid strains invariably form smooth colonies. Between these two extremes of rough, mucoid and smooth, non-mucoid there is the intermediate stage of smooth, mucoid colony which is usually in a state of transition.

Material and Methods. The cultures used in the work were those described in Part 1 of this bulletin. Most of the work was done with two strains, both isolated from foals dead at the age of 24 hours, and the variants derived from these strains. While the cultures used were not isolated from single cells they were derived from cultures which were apparently pure at the time they were isolated. These strains have been cultivated artificially from one to three years and during this period have been plated repeatedly. Some of the cultures, particularly the two strains 36 and 38, with which most of the work was done, have been plated 3 times each week for 2 years. At no time has any evidence of contamination been observed. It is felt that there can be no reasonable doubt as to the purity of these cultures.

In the present work the morphology, cultural and biochemical characters, and serological properties of the rough and smooth forms have been examined. In addition, the effect of certain environmental factors on the two forms, and conditions which tend to bring about the conversion of one form into

the other, were studied. Thruout the course of the work the substrates upon which the organisms have been cultivated were kept as nearly constant as possible. The broth used to cultivate the organisms was composed of one percent of Bacto peptone and 0.3 percent of meat extract, in tap water. The reaction was adjusted to pH 7.6. The agar used in transferring stock cultures and plating was prepared from fresh beef infusion and contained one percent of Bacto peptone and 2 percent of agar. The reaction was pH 7.6. Both the broth and agar were made up in large lots but since the work has covered a period of more than two years it has been necessary to use several lots of both mediums. Stock cultures were maintained on agar slants. They were transferred every sixth day, incubated at 37° C for 24 hours and then kept in the dark at room temperature.

Morphology of rough and smooth forms. The morphology of *S. equirulis* is quite variable. This lack of uniformity which has been emphasized by Goerttler,[17] extends to both the rough and smooth forms. However, from repeated examinations of a number of rough and smooth strains certain morphological differences are apparent in the two types. If agar cultures are examined at the age of 8 to 10 hours many unusual forms are found in both rough mucoid and smooth strains. Large yeast-like bodies bearing projections resembling buds are present. These extremely large forms take the ordinary stains very poorly. Long, filamentous forms and streptococcus-like chains are found in both rough and smooth forms at this age. However, chains and filamentous forms occur much more commonly in the smooth than in the rough forms.

At 14 to 16 hours of age the difference in morphology of the cultures is much more pronounced. At this time the rough, mucoid culture has assumed the morphological characters generally attributed to this species, the culture is almost entirely composed of short, oval rods. The smooth culture, on the contrary, will be found to abound in long, filamentous forms, streptococcus-like chains and very large, poorly-staining organisms. After 24 hours incubation the rough, mucoid culture is composed entirely of short rods, while filaments and chains are

still numerous in the smooth culture. Streptococcus-like chains and filamentous forms are much more common at all ages in the smooth than in the rough cultures.

Both the rough and smooth forms are non-motile. Capsule formation by either form has not been observed, altho the rough, mucoid form seems to produce an intercellular substance not found in the smooth form. This substance is stained poorly or not at all by the ordinary methods of staining. However, the rough, mucoid bacilli apparently are imbedded rather firmly in this substance and when placed in a slide are held together in groups or masses.

CULTURAL CHARACTERS OF THE ROUGH AND SMOOTH FORMS

Agar colonies. Rough form. Well isolated agar colonies attain considerable size, 3 to 5 mm., in 48 hours. These are raised, opaque, and extremely tenacious. At times the colonies are almost hemispherical. The edges are undulate and the surface is extremely rough. When examined under a hand lens by reflected light they present a lobulated appearance, the small, lobular projections appearing over the entire surface of the colony. Between the lobules deep crevices run irregularly over the surface. When viewed by transmitted light the roughened surface causes the colony to exhibit an irregular radially striated structure. With increasing age a clear, smooth border zone may be extended from the colony.

Smooth Form. The diameter of colonies of the smooth form is the same as that of the rough form, or slightly greater. They are flat or very slightly raised, translucent, and non-mucoid. The edges of the colonies are entire and the surface is perfectly smooth. Viewed by transmitted light the colonies present a homogenous structure. The colonies of the smooth form rather closely resemble those formed by the members of the colon-typhoid group.

Agar slants. The growth of the two forms on agar slants possess many of the characters of the agar colonies. The growth

of the rough form is more opaque, roughened and tenacious than that of the smooth form.

Growth in broth. Rough form. The first growth to become apparent is a collection of small masses of bacteria along the side of the tube. A few masses may also be observed floating on the surface of the liquid. As growth continues a light pellicle of uneven thickness is formed on the surface of the broth and the sides of the tube become covered with a deposit of the bacteria. A ropy sediment containing granules appears and eventually a diffuse growth occurs thruout the broth. Broth cultures of this form become very viscous.

Smooth form. The smooth form brings about an even clouding of the broth with little sediment and without pellicle formation.

Biochemical characters of rough and smooth forms. No differences have been demonstrated in the biochemical properties of the two forms, using the qualitative tests employed in these studies.

THE INFLUENCE OF CERTAIN ENVIRONMENTAL CONDITIONS ON ROUGH AND SMOOTH FORMS

Growth on Agar. Growth on the surface of agar is favorable to the development of the smooth form. Rough cultures transferred every sixth day for the maintenance of stock cultures, soon begin to lose their roughness and tenacity. After a few transfers the organisms are transformed to the smooth, mucoid variety. They continue to become less mucoid until they reach the smooth, non-mucoid form. The length of time required for this change varies from four to five weeks to more than a year. A few cultures in our possession have reached the smooth, mucoid state after a few transfers but on continued culture they have retained their mucoid characters. Other strains have changed from the rough mucoid form to the smooth, non-mucoid state and then reverted to the mucoid form. However, the majority of the strains have become non-mucoid and remained in this condition.

Growth in broth. The rough, mucoid form is much more

stable in broth than on the surface of agar. When rough, mucoid cultures are transferred daily in 10cc of broth the smooth types are much slower in appearing than when the same strains are kept on agar. The ability of strains to maintain themselves in the rough, mucoid form under these conditions varies. Strain 36 when treated in this manner gradually lost its roughness and was transformed to smooth, non-mucoid. Strain 38, on the other hand, continued to produce mucoid colonies although the rough character disappeared. Continued culture in broth without transfer leads to a more rapid appearance of the smooth form than does daily sub-culture in broth. *S. equirulis* does not remain viable in broth cultures for more than 18 to 21 days. Within this time smooth colonies begin to appear. Daily transfer or continued culture of smooth forms in broth is not sufficient to restore smooth forms to the rough state. Some strains under these conditions produce mucoid colonies but do not revert to the rough form.

Growth in culture filtrates. During the course of the work it was noted that when large numbers of colonies appeared on the plates of rough strains, the colonies were no longer rough but smooth. This suggested that the accumulation of metabolic products in cultures was responsible for the gradual change from rough to smooth in cultures of *S. equirulis.* Rough and smooth forms of strain 36 were cultivated in broth and the cultures sterilized by filtration. The rough and smooth forms of this culture were inoculated into the filtrates of both the rough and smooth cultures. No growth took place in any of the tubes. The filtrates were then added to uninoculated broth in the amount of 25 percent. Both the rough and smooth forms developed in the presence of 25 percent of the filtrates. The cultures were transferred daily in these mediums for 18 days and the 24-hour broth cultures plated daily. Under these circumstances the presence of the culture filtrates apparently had no effect on the type of colony produced by the organism.

Growth at different temperatures. In order to determine the effect of temperature on the colony form of *S. equirulis* rough and smooth forms of strain 36 were grown in broth at

30°, 37° and 42°. The broth cultures were transferred daily for 24 days. Plates were streaked from the 24-hour-old broth cultures. The plates were incubated at the same temperature as the culture with which they were inoculated. With the rough form, the culture incubated at 30° continued to produce rough colonies thruout the course of the experiment. No smooth colonies appeared in this culture. Smooth colonies began to appear in small numbers after the fourth transfer in the culture incubated 37°. These increased in number until the culture was producing only smooth, mucoid colonies at the end of the experiment. Smooth colonies appeared in the second transfer of the culture kept at 42°. These increased in number and the culture successively became less mucoid until at the end of the experiment only smooth, non-mucoid colonies were present on the plates.

The smooth, non-mucoid form of strain 36, when grown at 37° and 42°, continued to produce smooth, non-mucoid colonies. When grown at 30° C the colonies became mucoid after the second transfer. At the end of the experiment the colonies were still smooth but very mucoid. It is evident that lowered temperatures favor the rough, mucoid form.

Growth at different hydrogen ion concentrations. The rough form of strain 38 was planted in broth having the following hydrogen ion concentrations: pH 5.4, 6.0, 6.6, 7.2, 7.8, 8.0 and 8.4. The cultures were incubated at 37° and transferred daily. Plates were streaked from the 24-hour broth cultures on agar of the corresponding reaction. The experiment was continued for 18 days. No growth occurred in the broth having a reaction of pH 5.4. Growth was delayed and poor in the broth having a reaction of pH 6.0. On the corresponding plates the colonies that developed were small and few. After the third transfer these colonies became smooth and non-mucoid. The colonies that developed on the medium of pH 6.6 became smooth after the sixth transfer and toward the end of the experiment became non-mucoid. The remainder of the cultures produced rough, mucoid colonies thruout the experiment. The cultures grown at pH 7.8 and 8.4 were extremely rough and

tenacious. It is apparent that an acid reaction favors the change from rough to smooth. It has been stated previously that the strain used in this experiment, 38, has less tendency to grow in the smooth form than the other cultures studied. Had another strain been used in this work it is probable that the changes observed would have been more extensive.

Growth in the presence of different amounts of normal horse serum. Plain broth and broth containing 10, 25, and 50 percent of normal horse serum were inoculated with a culture of strain 36 which was producing rough and smooth colonies in approximately equal numbers. The cultures were transferred daily and plates streaked from the 24-hour broth cultures. The cultures were then corked to prevent evaporation and incubation was continued. After different periods these cultures were replated. Under these conditions normal horse serum exerted no influence on the type of colony produced.

Growth in the presence of antiserum. Many workers have been successful in changing the colony types of organisms by cultivating them in their specific antiserums. In the present work the organisms were grown in plain broth, broth containing 25 percent of normal rabbit serum, and broth containing 25 percent of specific antiserum from rabbits. The cultures were transferred daily for 24 days and the 24-hour broth cultures were streaked on plates. After plating, the tubes were corked to prevent evaporation and incubation was continued at 37°. After different intervals the cultures were replated. The cultures used in this work were the rough and smooth forms of strains 36 and 38. The smooth form of strain 36 was derived from the rough form thru growth in antiserum. The smooth form of strain 38 was isolated directly from the infected foal at post mortem.

The rough form of strain 36, when grown in the presence of antiserum, began to produce smooth, non-mucoid colonies after the third transfer. During the course of the experiment the number of these smooth colonies increased until the culture was producing approximately 50 percent smooth colonies. This change from rough, mucoid to smooth, non-mucoid took place

without the appearance of the intermediate smooth mucoid form. The culture grown in 25-percent normal rabbit serum continued to produce only rough colonies thruout the experiment. The cultivation of the smooth form of strain 36 and the rough and smooth forms of strain 38 in antiserum failed to bring about any change in the character of the colonies produced.

Growth in a large amount of broth. A smooth, non-mucoid culture of strain 36 was grown in 500cc quantities of broth and transferred daily. Plates were streaked from the 24-hour cultures. Colonies arising from the first six transfers resembled the original cultures. On the seventh transfer the colonies were noticed to be more mucoid and a few were decidedly more opaque than the original. There were two colonies on this transfer the surface of which was definitely roughened. The colonies from the eighth and ninth transfers resembled those of the seventh. A roughened colony that appeared on the ninth transfer was planted in 10cc of broth and plated after 24 hours. The colonies that appeared on this plate were a mixture of rough and smooth. The roughest colony was again selected and planted in broth. By continuing this process of selection a culture was obtained after six platings which had all the characters of the rough, mucoid type.

Altho the daily subculture in 500cc of broth was continued thru 35 transfers no further change was noted. The production of smooth, mucoid colonies continued with the occasional appearance of a few slightly rough colonies. This experiment was repeated with a number of strains. It was found that selection must always be resorted to to obtain a typical rough, mucoid culture. The method described is the only one by which we have been successful in obtaining rough cultures from strains which had become smooth.

Serological characters of rough and smooth forms. As shown in Part 1 of this Bulletin the serological characters of *S. equirulis* are not constant from strain to strain. A group of forty cultures studied was found to be hetergeneous and few antigenic relationships could be established. It is evident then that any study of the antigenic relationships of the rough and

smooth types must be carried out with the rough and smooth forms of the same strain or with strains known to be antigenically related. This was done, using both the rough and smooth forms of strains 36 and 38 to prepare agglutinating serums. The results of the agglutination tests are given in table 4.

Table 4. Agglutination of Rough and Smooth Variants.

Antigens	SERUMS			
	36 rough	36 smooth	38 rough	38 smooth
36 rough	2000	2000	0	0
36 smooth	2000	2000	0	0
38 rough	0	0	2000	2000
38 smooth	0	0	2000	2000
34 smooth	2000	2000	0	0
6 smooth	0	0	2000	2000

0 Indicates no agglutination at 1-20.

Table 4 shows that rough and smooth types are agglutinated reciprocally by rough and smooth antiserums. Strains 36 and 38 are not antigenically related and there is no cross agglutination by either their rough or smooth forms. Strain 34 was isolated as a rough strain but became smooth on continued culture. It is serologically identical with strain 36 and is agglutinated by both rough and smooth antiserums of that strain. Strain 6 was isolated as a smooth, non-mucoid type and has never produced rough or mucoid colonies since its isolation. It is serologically identical with strain 38 and is agglutinated by both rough and smooth antiserums for that strain. No constant differences in the agglutinability of the rough and smooth forms were noted. Spontaneous agglutination occurred in certain strains but this had no relation to the rough or smooth type.

In testing the ability of the variants to absorb agglutinins it was found that the smooth form of strain 36 completely exhausted the rough antiserum of agglutinins for the rough form. Likewise the rough form completely removed the agglutinins from smooth antiserum for the smooth form. The same

relationships were found to exist between the rough and smooth forms of strain 38. Strain 34 (smooth) completely removed the agglutinins from 36 rough and 36 smooth antiserums. Strain 6 (smooth) absorbed the agglutinins from both the rough and smooth antiserums of strain 38.

It is a significant fact, however, that a much larger amount of the smooth organisms than of the rough was required to remove the agglutinins from rough antiserum. When smooth bacteria were used to absorb a rough antiserum the absorbing dose had to be increased 8 to 10 times in order to remove agglutinins completely. The explanation of this, we believe, is given in the following paragraph.

Production of specific substances by rough and smooth forms. S. equirulis produces a soluble specific substance which can easily be detected in broth cultures and in the filtrates of autoclaved saline suspensions. Such filtrates serve well as antigens in the precipitin test. In order to determine the relative amounts of specific substances produced by rough and smooth forms saline suspensions of the rough and smooth forms of strain 36 were prepared. The turbidities were equalized and the suspensions autoclaved at 15 lbs. pressure for 15 minutes. The suspensions were cleared by Berkefeld filtration and used as antigens in precipitin tests. Both rough and smooth antiserums were used in these tests but since the results were nearly identical only those obtained with the rough antiserum are given in table 5.

Table 5. Production of Specific Substances by Rough and Smooth Type.

Amount of Antigen	Degree of Precipitation		
	36 Rough	36 Smooth	Control
1.0	++++	++++	—
0.5	++++	++++	—
0.3	++++	+	—
0.1	+++	—	—
0.05	+++	—	—
0.01	++	—	—

0.1cc 36 rough serum used in all tests.

It is evident that the rough, mucoid culture produced a much larger amount of specific substance than the smooth, non-mucoid culture. The filtrate from the rough culture caused marked precipitation when .01cc was used, whereas the smooth form failed to cause precipitation when 0.1cc was used in the test. The small production of specific substance by the smooth form may be the reason why an increased amount of the smooth antigen is necessary to absorb the agglutinins from rough anti-serum.

Virulence of rough and smooth forms. The estimation of virulence of cultures of *S. equirulis* is rather difficult since it is non-pathogenic for laboratory animals. Adult horses, also, are quite resistant to the organism. Only foals a few days old possess a high degree of susceptibility. Since it was impossible to obtain such foals it was necessary to confine our observations to the production of local reactions in older horses. This method is admittedly unsatisfactory.

The practice has been to inject subcutaneously the rough and smooth forms of the same strain into the same animal at different sites and to observe the effect produced. Under these circumstances no difference in the virulence of the two forms could be noticed. Both forms produced subcutaneous abscesses which ruptured and discharged a purulent exudate. After rupture the abscesses healed slowly. A marked difference was noted in the virulence of recently isolated strains and strains which had been under culture for some time. Recently isolated cultures not only produce local reactions in adult horses, but cause a general reaction with increased temperature, increased pulse rate, general depression and stiffness of the joints. As culture is continued the organisms progressively lose virulence regardless of whether they are maintained in the rough or smooth form. The fact that both the rough and smooth forms have been isolated in pure culture from the tissues of infected foals indicates that both are virulent for very young horses.

Variation in vivo. The fact that rough and smooth forms of *S. equirulis* sometimes are observed in the same foal indicates that variation may occur in the body of the infected individual.

In an effort to determine whether such variation does occur pus was aspirated from each of the abscesses produced in determining the virulence of the organisms. This pus was plated immediately and planted in broth and the broth cultures plated after 24 hours incubation at 37°. No variation in the colony forms of the injected cultures was observed in these tests. Rough cultures always were recovered when rough cultures were injected. When smooth cultures were administered the colonies appearing on the plates were of the smooth variety. We have been unable to demonstrate any change in the character of the organisms in the body of the horse.

Isolation of smooth type from foals. The recognition of the smooth, non-mucoid type as *S. equirulis* is of importance since it sometimes is isolated directly from the tissues of infected foals. It may be found associated with the rough, mucoid type or may be present alone. Within the past four years 88 foals infected with *S. equirulis* have been examined. In five cases only the smooth form of the bacterium was present. In many other cases the smooth form was found associated with the rough form.

That these organisms were in reality *S. equirulis* can hardly be doubted. Although the heterogeneous antigenic characters of this species make its identification rather difficult, all these forms possessed the cultural and biochemical properties generally attributed to this organism. All the smooth forms lived for only short periods on the surface of agar, a character typical of *S. equirulis.* In addition, some of the smooth forms have been shown to be closely related antigenically to certain rough, mucoid strains. Some of the cultures which were smooth when isolated were transformed to the rough, mucoid type thru growth in large amounts of broth and selection of colonies. Other strains have remained smooth and non-mucoid in spite of attempts to cause them to become rough.

DISCUSSION

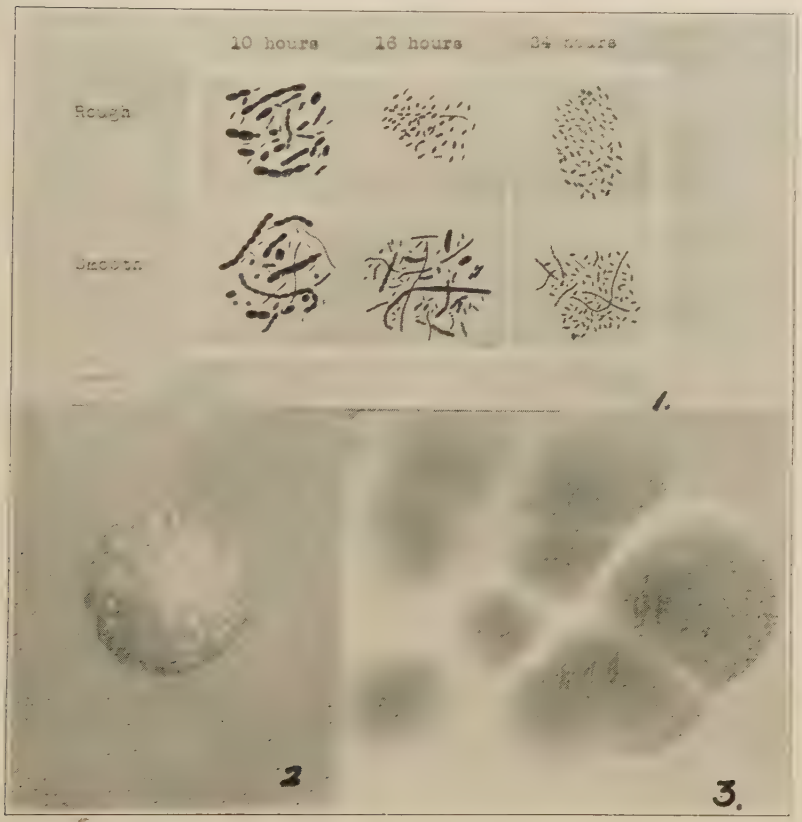
The existence of mucoid and non-mucoid strains of *Shigella equirulis* has been shown to be closely related to the phenomenon

of rough and smooth variation. Rough cultures invariably have been found to be mucoid. Non-mucoid cultures always produce smooth colonies. In cultures which have been isolated for some time a smooth mucoid form is found. This intermediate stage between the two extremes may arise only in artificial cultures, at least it has never been observed in primary cultures from infective material. This form, if it ever occurs naturally in the animal body, is rare. The rough mucoid form seems to be the normal type of the organism, since it is isolated much more frequently than the smooth, non-mucoid form.

The trend of variation in this species is from rough to smooth. While certain environmental conditions have been found to influence the type of colony produced, it has not been possible to control variation in this species regularly. The stability of the rough form varies greatly from strain to strain. Some strains, for example, 38, can be maintained in the rough form without difficulty, whereas others are very difficult to keep in the rough form. The stability of the rough form may vary also in the same strain at different times. A culture which has been producing rough colonies regularly may suddenly begin to give rise to colonies which are less rough and more moist. The strain may then begin to produce extremely rough colonies again.

If a culture is to be maintained in the rough state indefinitely it is necessary to practice selection constantly. The only method found satisfactory in keeping rough cultures is to cultivate the organisms in broth for 24 hours, plate the broth culture and select the roughest colony appearing on the plates to fish back to broth. Unless continued selection is resorted to the rough form cannot long be maintained. In this species the colonies possess all degrees of roughness and the change from rough to smooth and from mucoid to non-mucoid is a gradual one.

While some workers, notably Goerttler,[17] have denied that a non-mucoid form of *S. equirulis* exists, the results obtained in the serological studies should fix beyond doubt the identity of the smooth, non-mucoid cultures being studied. It was shown



1. Camera lucida drawings of rough and smooth variants of Shigella equirulis at different stages of incubation.
2. Rough colony S. equirulis, 48 hours incubation, x 4.
3. Smooth colony S. equirulis, 48 hours incubation, x 4.

that these cultures were very closely related and capable of the mutual absorption of agglutinins. This fact, combined with the identity of their biochemical reactions, clearly indicates that they are members of the same species. Smooth, non-mucoid forms were found to produce a smaller amount of soluble specific substance than rough, mucoid forms. However there was never encountered a culture in which the production of specific substance was entirely suppressed.

Part 3. The Occurrence of Dwarf Colony Variants.

The occurrence of variants forming dwarf colonies is unusual and few reports of such organisms are to be found in the literature. Jacobsen[24] isolated a culture of *Eberthella typhi* the growth of which was greatly restricted. This organism was isolated from a blood culture and grew in very small streptococcus-like colonies on nutrient agar. The bacterium exhibited the usual physiological properties of *E. typhi* except that it produced late fermentation in mannite broth. The culture was inagglutinable. When the organism was streaked on Conradi-Drigalski plates only very tiny, streptococcus-like colonies were visible after 48 hours incubation. On the third day large colonies appeared among the small colonies. These large colonies gave rise to typical races of *E. typhi* which were readily agglutinable in antiserum. The growth of the dwarf variant was stimulated by the addition of blood, serum, or ascitic fluid to the medium. It grew in large colonies on Endo's medium, due to the sodium sulfite ($Na_2 SO_3$) content of the agar. Jacobsen was able to demonstrate that the restricted growth of this variant was due to the autoclaving of the culture medium. On agar which was autoclaved for 30 minutes in its preparation the bacterium grew in dwarf colonies, while on agar which was autoclaved for only 15 minutes it grew as normal *E. typhi.*

Fromme[25] isolated a strain of *E. typhi* which at first grew typically but on continued transfer produced small, streptococcus-like colonies. The characters of this culture were much like those of the organism described by Jacobsen.[24] It differed in that it was agglutinable, never gave rise to large-colony forms, and did not grow in large colonies on agar which had been heated for only a short time. The bacterium was found

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[24] Jacobsen, K. A. 1910. Mitteilungen über einen variablen Typhusstamm (Bakt. typhi mutabile) sowie ueber eigentümlichen hemmende Wirkung des gewöhnlichen Agar, verursacht durch Autoklavierung. Centbl. Bakt: (Etc.), I, Orig. *56*, 208.

[25] Fromme. 1911. Ueber einen atypischen Typhusstamm. Centbl. f. Bakt. (Etc.), I, Orig. *58*, 445.

to be antigenically identical with E. typhi by reciprocal absorption tests.

Eisenberg[26] described a variant of *Serratia marcescens* which formed dwarf colonies. Eisenberg also described two dwarf variants of *E. typhi* recovered from old blood-broth and blood-bile cultures. The first variant was inagglutinable but gave rise to agglutinins for *E. typhi.* Large-colony-forming strains occasionally appeared in cultures of the variant. Unlike the dwarfs described by Jacobsen and Fromme the growth of this variant was not stimulated by the presence of blood, serum, ascitic fluid or sodium sulfite (Na_2 SO_3) in the medium. The second variant described by Eisenberg was much like the first except that large-colony-forming strains never appeared in the culture and it did not give rise to agglutinins for E. typhi. Eisenberg considers this an even more pronounced degeneration than the first form.

Fürth[27, 28] described dwarf variants of *Salmonella aertrycke* and *Para-typhus beta* (Weil). These organisms were isolated from old broth cultures. Both organisms fixed complement in the presence of antiserum of their respective parent strains and both gave rise to large-colony-forming strains.

Occurrence. In the post-mortem examination of foals a number of cultures of *S. equirulis* in which growth was greatly restricted were isolated. During the past 5 years 88 foals infected with *S. equirulis* have been received in the laboratory. Attention was not drawn to the dwarf forms until the latter part of this period so that an accurate record of the occurrence of these strains is available only for the past 2 years. During these two years the dwarf variants, either alone or in association with the normal form, occurred in 25 percent of the foals examined. The variants can be cultivated directly from the infected animal. Their occurrence may be confined to cultures from one organ or they may appear on cultures from all the

[26] Eisenberg, P. 1914. Untersuchungen über die Variabilität der Bakterien. Centbl. Bakt. (Etc.), I, Orig. *73*, 449.

[27] Fürth, J. 1922. Variationsversuche mit Paratyphus beta (Weil). Zeitschr. Immunitätsf. u. Expt. Ther., *35*, 155.

[28] Fürth, J. 1922. Rezeptorenanalyse und Variationsversuche mit B. paratyphus Aertryck. Zeitschr. Immunitätsf. u. Expt. Ther., *35*, 162.

organs. In two instances dwarf forms were isolated from the uterine discharges of mares that had given birth to infected foals. In four cases the variants appeared in stock cultures of *S. equirulis* which had previously possessed characteristics typical of this species. This change from the normal form to the dwarf did not occur gradually but took place suddenly, changing completely as the cultures were transferred. The length of time elapsing between isolation and change of the cultures varied from 2 to 13 months. These cultures originated from single-colony isolations and had all been plated several times prior to the change to the dwarf form. No dwarf colonies were observed previous to this change and there was no reason to doubt the purity of the cultures.

Morphology. The dwarf cultures, when examined under the microscope, were found to be composed largely of short, oval rods. Occasional filamentous forms and streptococcus-like chains were present. The organisms closely resembled those found in normal cultures of *S. equirulis.*

Cultural and biochemical characters. The difference in appearance of the normal and dwarf colonies is most striking. While the normal colonies attain a diameter of 3 to 5 millimeters in 48 hours and are usually quite opaque the dwarfs form very small, transparent, streptococcus-like colonies, .1 to .25 millimeter in diameter after, 96 hours incubation. The variations occurring in the normal strains which result in the appearance of rough, smooth, mucoid, and non-mucoid colonies are apparent also in the dwarf races and the characters of the dwarfs correspond closely to those of the normal types with which they are associated or from which they are derived. When the dwarf strains are isolated directly from infective material their characters are the same as those possessed by the normal strains recovered from the same source. When rough, mucoid colonies are isolated from a foal the dwarf colonies that appear on the cultures are of the rough, mucoid variety. If smooth, non-mucoid colonies are isolated, the dwarfs are smooth and non-mucoid. In the instances in which the dwarf forms replaced normal forms in stock cultures their characters, as regards

roughness and viscosity, were the same as those of the strains from which they were derived. It was stated in Part 2 of this Bulletin that one of the most prominent characters of *S. equirulis* is its inability to survive for more than a few days on the surface of agar. The dwarf variants also possess this character, remaining viable for about the same length of time as the normal forms.

In contrast with normal strains, which grew readily in beef extract peptone broth, the majority of the dwarf forms failed to multiply in this medium. A few of the variant strains produced a slight growth in broth which was evident only by a faint clouding of the liquid after 4 or 5 days incubation. The dwarf strains also grew poorly in litmus milk. Some failed to produce any change in this medium while others produced a faint acidity. Tests for the reduction of nitrate and the production of ammonia also gave irregular results because of the failure of many of the dwarfs to grow in the medium used in the tests.

The same difficulty was encountered in determining fermentative characters. Results of fermentation tests were extremely irregular and where acid production was observed it was delayed and slight. This difficulty was overcome by the addition of 10 percent of sterile normal serum to the basic medium used in the tests. The addition of fresh blood serum greatly stimulates the growth of the dwarf forms and in its presence they exhibit fermentative characters identical with those of the normal type.

Serological reactions. It was shown in Part 1 that cultures of *S. equirulis* do not conform to a single serological type but vary in their antigenic composition. It is evident that any attempt to demonstrate a serological relationship between the dwarf variants and normal strains must be based on a comparison of cultures derived from the same source. In studying the serological characters of the dwarf strains our practice has been to select cultures from cases which on post-mortem examination yielded both normal and dwarf strains. A normal culture and a dwarf culture from such a case were used to prepare agglutinating serums and these serums were used to determine antigenic relationships. In this way it is possible to

demonstrate that certain of the dwarf variants are closely related to normal strains derived from the same foal. In table 6 the agglutination reactions of two dwarf strains and two normal strains are given.

Table 6. Agglutination reactions.*

Antigens	Serums			
	18 Normal	18 Dwarf	36 Normal	36 Dwarf
18 Normal	2000	2000	20	20
18 Dwarf	2000	2000	20	20
36 Normal	20	20	2000	1000
36 Dwarf	20	20	2000	2000

* Figures indicate highest dilution at which agglutination occurred.

From table 6 it is seen that each of the two dwarf strains is related to the normal strain derived from the same source.

The precipitin reaction may also be used to demonstrate these serological relationships. The method used was that described in Part 1. The precipitin reactions of a normal culture and a dwarf culture associated with it are given in table 7.

By the use of reciprocal absorption tests it is demonstrated that strain 36 normal effects a complete removal of agglutinins from serum derived from strain 36 dwarf. Likewise strain 36 dwarf completely absorbs agglutinins from serum derived from strain 36 normal.

Using this method, five dwarf strains and their corresponding normal strains were examined. Two of these were found to be antigenically identical with the normal strains with which they were associated. The other three showed no serological relationships.

That certain of these strains have been found to be antigenically identical with normal cultures of *S. equirulis* should remove all doubt as to the identity of these dwarf variants.

Virulence. Normal strains of *S. equirulis* are known to be non-pathogenic for the laboratory animals. The dwarf variants are also without effect when injected into rabbits, guinea pigs,

and rats. When injected intravenously into old horses they bring about the same changes as do normal strains. Introduction of the organisms is followed by a rise in temperature, increased pulse rate, general depression, and stiffness of the

Table 7. Precipitin Reactions.

Serums	Amount cc	Antigens: Degree of Precipitation			
		36 Normal	Control	36 Dwarf	Control
36 Dwarf 0.1 cc	1.0	++++	—	++++	—
	0.5	++++	—	++++	—
	0.2	+++	—	+++	—
	0.1	+++	—	++	—
	0.03	+	—	+	—
36 Normal 0.1 cc	1.0	++++	—	++++	—
	0.5	++++	—	++++	—
	0.2	++++	—	+++	—
	0.1	+++	—	++	—
	0.03	++	—	+	—

0.1 cc 36 rough serum used in all tests.

joints. These symptoms persist from 2 to 7 days and are followed by complete recovery. We have been unable to observe the effect of injection of these dwarf forms into very young foals because of lack of experimental animals. However, the fact that dwarf strains in pure culture are isolated from fatal cases of natural infection indicates that they possess the same virulence as normal strains of the organism.

Occurrence of large-colony-forming strains in cultures of dwarf variants. Large-colony forms have appeared in the majority of the dwarf variants described by other workers. Twelve dwarf variants of *S. equirulis* were kept in the laboratory for study. Six of these have been under artificial cultivation for 30 months and the remaining six for more than 18 months. During this time only one dwarf culture was observed to produce large colonies. This culture was isolated as a dwarf

and during the first 4 months it was under cultivation produced only tiny, streptococcus-like colonies. During this four months the stock culture was transferred 20 times without the appearance of large colonies. On the twenty-first transfer several large, raised, opaque colonies appeared on the thin, transparent growth covering the agar slant. Transfers from the large colonies yielded only large colonies. This large-colony-producing strain was subjected to repeated plating. No dwarf colonies were observed in this culture. Transfers were made, also, from the dwarf growth present on the mixed culture. These transfers after 24 hours incubation showed only dwarf colonies. After standing 4 days at room temperature large colonies appeared on the dwarf growth. This culture was plated repeatedly, small colonies being selected for transfer each time. For the first few platings large colonies appeared on the plates but after continued selection the culture ceased to produce large colonies and remained a pure dwarf culture for 10 months, after which time it again began to produce a mixture of large and small colonies. Agglutinating serums were prepared for the dwarf strain and for the large colonies which had developed from it. Serological reactions showed them to be antigenically identical. The large-colony-forming strain which originated from the dwarf culture possessed all the properties of a typical culture of *S. equirulis.*

Stimulation of dwarf variants. Most workers who have described dwarf variants have found that their growth was stimulated greatly by the addition of certain substances to the medium. This is true of the dwarf varieties of *S. equirulis.* It was found that the addition of blood, blood serum, ascitic fluid, cabbage juice or potato juice caused these dwarf strains to form colonies as large as those of the normal strains of the organisms. Not only were these colonies comparable in size with those of the normal strains but they resembled them closely in appearance. As stated earlier the dwarf strains, like the normal strains, exhibit rough and smooth variation. When these dwarf strains are grown upon a medium containing one of these stimulating substances the rough strains produce large, rough, opaque, tenacious colonies. The smooth strains under the same condi-

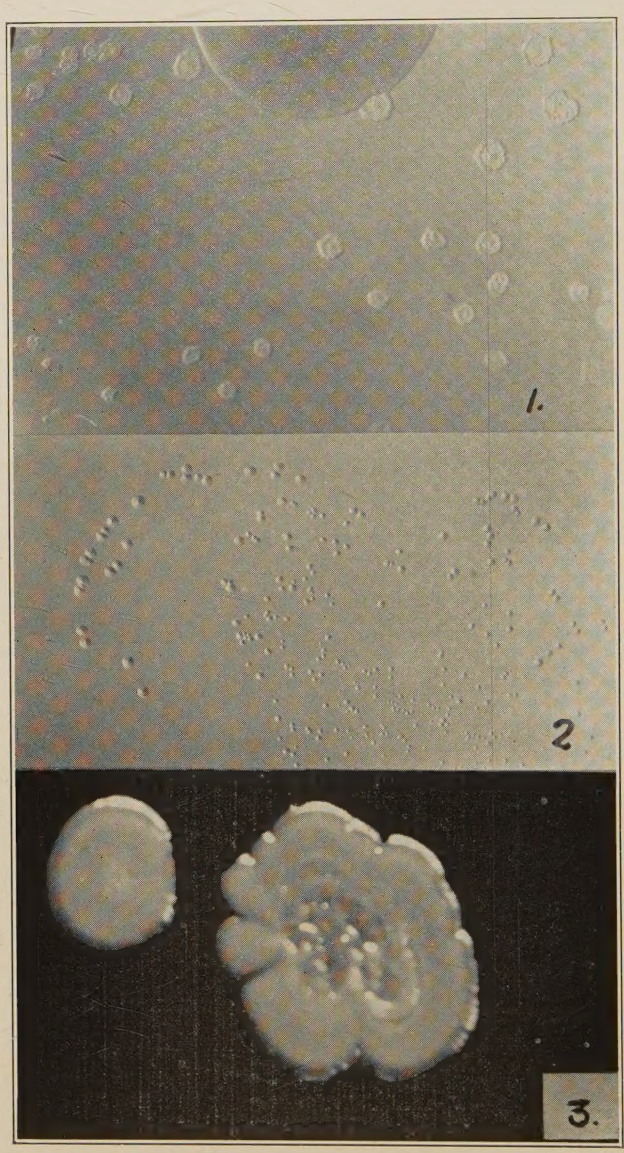
tions produce large colonies which are less raised, smooth, translucent and non-tenacious. It is impossible to distinguish a dwarf culture growing upon a medium containing a stimulating substance from normal strains of the organism.

Jacobsen[24] and Fromme[25] reported that their dwarf varieties of *E. typhi* were stimulated by various inorganic compounds, notably sodium bisulfite ($NaHSO_3$) and ammonium hydrosulphide (NH_4SH). The dwarf varieties of *S. equirulis* were not influenced by these substances. Tho blood serum stimulated the organisms, washed blood cells did not. An attempt was made to determine what constituents of blood serum and cabbage juice were responsible for the stimulation, by fractionating the proteins by precipitation with ammonium sulfate. The stimulating principle evidently was not precipitated with the proteins of these substances, since these proteins, when precipitated, redissolved and added to the medium, were without effect. The effect of the residue left after precipitation of the proteins could not be determined since the ammonium sulfate content inhibited the growth of the organisms.

Fresh meat infusion also stimulates the growth of the dwarf strains. These growth-stimulating principles are quite heat stable. Cabbage juice and meat infusion autoclaved at 15 pounds pressure for 4 hours were still capable of stimulating growth. When autoclaved for 6 hours this property is lost. Meat extract peptone mediums have no stimulating properties even when heated for only 10 minutes. On meat extract mediums the dwarf strains grew very poorly, their colonies being barely visible after 24 hours incubation. The normal strains, on the contrary, grew quite well. That the dwarf strains grow in small colonies on the stock meat infusion agar used in this work probably is due to the agar having been made up in very large lots and hence subjected to prolonged periods of heating.

SUMMARY

The cultural, morphological, biochemical, and serological properties of 40 cultures of *Shigella equirulis* were studied. The presence of extensive variations in the cultural characters of



1. Dwarf colonies of S. equirulis, rough form, 96 hours incubation, x 8. Large colony is contaminant.
2. Dwarf colonies of S. equirulis, smooth form, 96 hours incubation, x 4.
3. Dwarf colonies of S. equirulis on blood agar, showing stimulating effect of medium, 48 hours incubation, x 8.

the bacterium were noted and described. All the cultures were found uniformly pleomorphic. The cultural characters of all the strains were similar, altho the organisms exhibited some variation in these characters, due to dissociation. The biochemical reactions of the organisms were comparatively uniform. Slight differences were noted in the fermentative reactions and the ability of the various cultures to coagulate milk. The organisms were extremely diverse in their serological characters. Using seven agglutinating serums, only three cases of identity were noted in the 40 strains. The most distinctive character of the organism is its inability to survive for more than ten days on the surface of an agar medium, when kept in the dark, at room temperature.

The presence of rough and smooth variation was apparent thruout the course of the work. Changes in the mucoid character of the organism also were noted and it was demonstrated that the change from mucoid to non-mucoid is associated with the change from rough to smooth. Rough colonies are always mucoid, while non-mucoid colonies are always smooth. There is a transitional stage of smooth mucoid. The trend of the variation in artificial cultures is from rough to smooth. The rate at which the change from rough to smooth occurs varies from strain to strain and may vary within the same strain at different times. The rough form can be perpetuated in cultures only by continued selection. Growth in broth, incubation at lower temperature, and alkaline reaction favor the development of the rough form. Growth on agar, incubation at a temperature above 37°C, and acid reaction accelerate the change from rough to smooth. The two forms are closely related serologically. The rough form produces a larger amount of specific substance than the smooth form. Both rough and smooth cultures were isolated directly from the tissues of diseased foals, either alone or associated.

In addition to rough and smooth dissociation another type of variation resulting in the formation of dwarf colonies was observed. These dwarf races, while differing markedly in their cultural behavior, are morphologically and biochemically iden-

tical with normal strains of *S. equirulis.* Certain of these variants were shown to be serologically identical with normal strains derived from the same source. The rough and smooth and mucoid and non-mucoid dissociation occurring in normal cultures was observed in the dwarf strains. Furthermore, the characters of the dwarf cultures as regards roughness or smoothness and the presence or absence of the mucoid state, were the same as the characters of the normal strain with which they were associated or from which they were derived. The growth of the dwarf variants could be stimulated greatly by the addition of blood serum, ascitic fluid, cabbage juice, or potato juice to the medium. Fresh meat infusion also stimulated the growth of the dwarf strains. The stimulating substances are quite heat stable, resisting autoclaving for 4 hours.

Dwarf strains were recovered from stock cultures of normal strains and directly from infected foals. Rough mucoid and smooth non-mucoid strains also were isolated directly from the animal body. The recognition of these variants is of importance in the diagnosis of the disease.